Enhancement of tumor invasion depends on transdifferentiation of skin fibroblasts mediated by reactive oxygen species

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Summary

Myofibroblasts, pivotal for tumor progression, populate the microecosystem of reactive stroma. Using an in vitro tumor-stroma model of skin carcinogenesis, we report here that tumor-cell-derived transforming growth factor β1 (TGFβ1) initiates reactive oxygen species-dependent expression of α-smooth muscle actin, a biomarker for myofibroblastic cells belonging to a group of late-responsive genes. Moreover, protein kinase C (PKC) is involved in lipid hydroperoxide-triggered molecular events underlying transdifferentiation of fibroblasts to myofibroblasts (mesenchymal-mesenchymal transition, MMT). In contrast to fibroblasts, myofibroblasts secrete large amounts of hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6), resulting in a significant increase in the invasive capacity of tumor cells. The thiol N-acetyl-L-cysteine, the micronutrient selenite as well as selenoprotein P and the lipid peroxidation inhibitors α-tocopherol and butylated hydroxytoluene significantly lower both the number of TGFβ1-initiated myofibroblasts and the secretion of HGF, VEGF and IL-6, correlating with a diminished invasive capacity of tumor cells. This novel concept of stromal therapy, namely the protection of stromal cells against the dominating influence of tumor cells in tumor-stroma interaction by antioxidants and micronutrients, may form the basis for prevention of MMT in strategies for chemoprevention of tumor invasion.

Key words: Myofibroblast, Reactive oxygen species, Transforming growth factor β, Tumor invasion, Tumor-stroma interaction

Introduction

Tumor progression is characterized by local accumulation of extracellular matrix components and connective tissue cells surrounding the tumor cluster, a phenomenon called tumor-stroma interaction (Bhowmick and Moses, 2005; Liotta and Kohn, 2001; Zigrino et al., 2005). The stroma is composed of inflammatory cells, small vessels, fibroblastic and myofibroblastic cells, and the disturbance of stroma constitutes the desmoplastic reaction, suggested to be essential in development of the invasion process (de Wever and Mareel, 2003). In melanoma and carcinoma, a wide variety of different cytokines and growth factors (e.g. transforming growth factor β1 or TGFβ) are expressed by tumor cells and stromal cells that promote neovascularization and tumor growth as well as migration during tumor invasion (de Wever and Mareel, 2003; Lazar-Molnar et al., 2000; Liotta and Kohn, 2001).

One of the cellular components in the stroma reaction is the myofibroblast, a modulated fibroblast that has acquired the capacity to express the biomarker α-smooth muscle actin (αSMA) and to synthesize large amounts of collagen and other extracellular matrix components (Kunz-Schughart and Knuechel, 2002). Myofibroblasts are beneficial during wound healing (Mori et al., 2005; Peters et al., 2005) but also are involved in disease states such as pulmonary fibrosis (Willis et al., 2005) or chronic renal disease (Yang and Liu, 2001). Myofibroblasts interact with epithelial cells and other connective tissue cells and may thus control such phenomena as tumor invasion and angiogenesis (Desmouliere et al., 2004). In that context, myofibroblasts are located at the tumor border, near the invasion front in colorectal cancer (Nakayama et al., 1998) and in various benign and malignant salivary gland neoplasms (Soma et al., 2001).

Although a role of inflammatory cells and endothelial cells in tumor immunity and angiogenesis has been described (Coussens and Werb, 2002; Folkman, 2002), the molecular events underlying the fibroblast-to-myofibroblast transition (transdifferentiation) as well as the tumor-invasion-promoting effect of myofibroblasts are not yet known. Recently, we showed a paracrine effect of tumor-cell-derived TGFβ1 on downregulation of gap junctional intercellular communication between stromal fibroblasts, dependent on generation of reactive oxygen species (ROS) (Stuhlmann et al., 2003; Stuhlmann et al., 2004).

Here, we examine the potential involvement of reactive oxygen species in mesenchymal-mesenchymal transition (MMT) of human dermal fibroblasts to myofibroblasts. We addressed the question of whether intervention with antioxidants and micronutrients may affect this process and the invasive capacity of a skin-derived squamous cell carcinoma cell line. Upon treatment with TGFβ1, the intracellular ROS

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level was increased through a protein kinase C (PKC)-dependent pathway. The elevated ROS level, assessed here as lipid hydroperoxides (LOOH), initiates a signaling process resulting in both MMT and release of proinvasive signals that promote tumor progression. In that context, we describe a significant increase in the invasive capacity of tumor cells using a filter-based in vitro invasion assay. Preincubation of fibroblasts with antioxidants lowered growth-factor-initiated lipid peroxidation, subsequently resulting in inhibition of αSMA expression and the appearance of the myofibroblastic cell type. To our knowledge, this is the first report linking an increase in lipid peroxidation products and potential intervention by antioxidants in stromal cells to the invasive behavior of the tumor cell.

Results

TGFβ1-mediated transition of fibroblasts to myofibroblasts

We studied the tumor-cell-initiated and TGFβ1-dependent expression of αSMA in an in vitro cell culture model of human dermal fibroblasts (HDFs) and the squamous carcinoma cell line SCL-1. Subconfluent fibroblast monolayer cultures in control conditioned medium (CM^{HDF}) for 5 days showed single cells with a myofibroblastic phenotype. When HDF and SCL-1 cells were co-cultured for 5 days ($\text{co-culture}^{HDF,SCL}$), there was a 75-90% increase in αSMA-positive cells, representing myofibroblasts (Fig. 1A). This also occurred when HDFs were treated with 10 ng/ml TGFβ1 in control conditioned medium ($CM^{HDF,TGF}$) for 2 days. Similarly, HDFs grown for 3 days with conditioned medium from tumor cells (CM^{SCL}) showed myofibroblastic phenotype and morphology. Treatment of HDFs with CM^{SCL} plus 5 μg/ml anti-TGFβ1 ($CM^{SCL,antiTGF}$) almost completely abrogated TGFβ1-dependent αSMA expression (Fig. 1A).

In addition, the αSMA protein levels were measured at 24 or 48 hours after treatment with different stimuli. Treatment of HDFs with recombinant TGFβ1 resulted in an up to 7.5-fold increase of the αSMA protein level at 24 hours after treatment compared with the untreated control (Fig. 1B). Concentration-dependent expression of αSMA was detected for rTGFβ1 (1-10 ng/ml) at 24 or 48 hours after treatment (data not shown).

Reactive oxygen species mediate TGFβ1-initiated αSMA expression

TGFβ1 and supernatants of SCL-1 tumor cells increase the intracellular ROS level of HDFs (Stuhlmann et al., 2004). Therefore, we addressed the question of whether ROS modulate induction of αSMA. Again, a significant increase in TGFβ1-initiated αSMA protein levels was detected compared with mock-treated controls (Fig. 2A). By contrast, N-acetyl-L-cysteine and selenite either completely prevented (NAC) or significantly lowered (selenite) the TGFβ1-triggered upregulation of αSMA protein levels 48 hours after treatment with the growth factor. In that context, rTGFβ1-initiated expression of αSMA was lowered by 62% upon treatment with selenite. Incubation of HDFs with the antioxidants alone did not affect αSMA expression compared with untreated controls (Fig. 2A).

Two independent in vivo studies, using selenoprotein P (SeP) knockout mice, showed that SeP transports selenium from the liver to various other organs, tissues and cells (Hill et al., 2003; Schomburg et al., 2003). The effect of SeP in lowering the growth-factor-mediated transdifferentiation of skin fibroblasts was studied. Subconfluent fibroblast

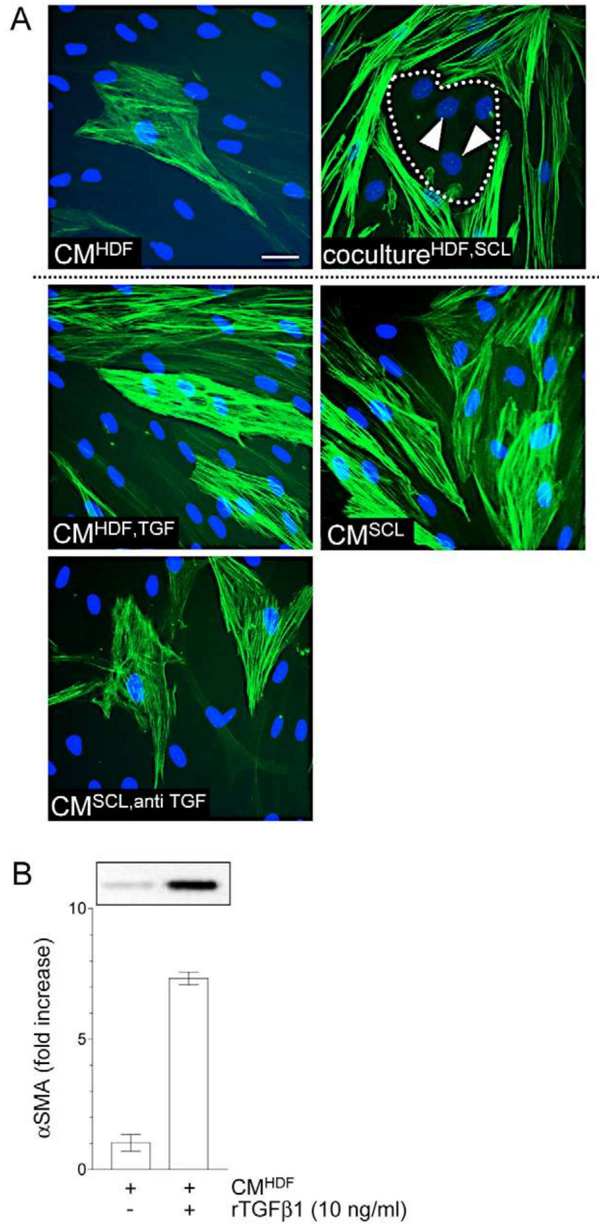


Fig. 1. TGFβ1-mediated transition of fibroblasts to myofibroblasts. (A) Subconfluent HDFs were cultured in control conditioned medium (CM^{HDF}), in co-culture with SCL-1 tumor cells ($\text{co-culture}^{HDF,SCL}$), treated with 10 ng/ml rTGFβ1 in CM^{HDF} ($CM^{HDF,TGF}$), treated with conditioned medium of SCL-1 cells (CM^{SCL}) or treated with CM^{SCL} containing 5.0 μg TGFβ1-neutralizing antibody ($CM^{SCL,anti\ TGF}$) for different time periods and then immunostained for αSMA. Representative images are shown. Dotted line highlights the tumor cell cluster, the arrows indicate the nuclei of the tumor cells. Bar, 20 μm. (B) Subconfluent HDFs were treated with 10 ng rTGFβ1/ml CM^{HDF} for 24 hours. The level of αSMA protein was determined by western blot. The densitometric values represent the fold increase over control, which was set at 1.0. The data represent means ± s.e.m. of three independent experiments.

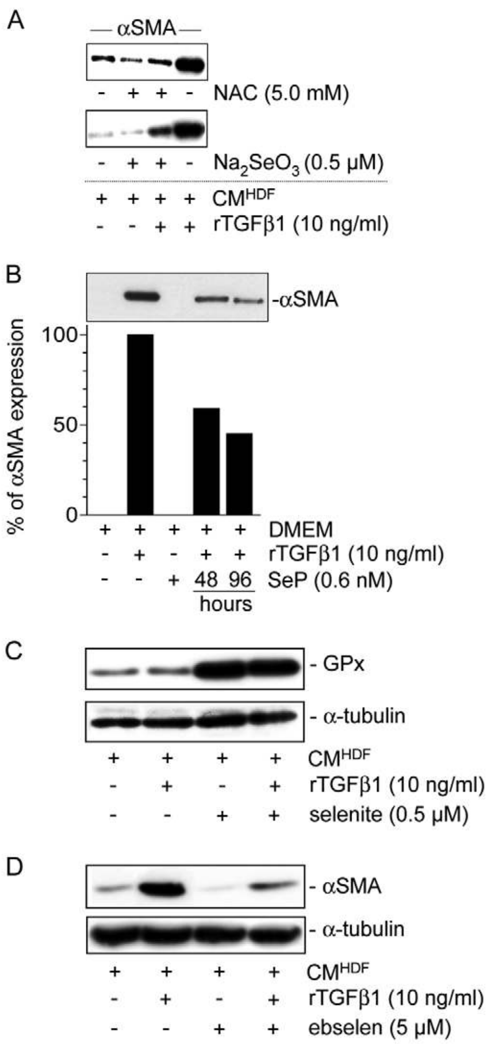


Fig. 2. Antioxidants downregulate TGFβ1-mediated expression of αSMA. (A) Subconfluent HDFs were cultured in CM^{HDF} and either untreated or pretreated for 4 hours with 5.0 mM NAC or for 24 hours with 0.5 μM Na_2SeO_3 before addition of 10 ng/ml rTGFβ1. TGFβ1 and the antioxidants were present for an additional 48 hours. The level of αSMA protein was determined by western blot. Three independent experiments were performed. (B) HDF monolayer cultures were cultured in CM^{HDF} either containing 0.6 nM SeP for 48 hours before treatment with 10 ng/ml TGFβ1 for a further 48 hours or containing SeP for the complete time of 96 hours. The level of αSMA protein was determined by western blot. The densitometric analysis describes protein expression as a percentage, setting rTGFβ1-treated controls at 100%. The experiments were performed in duplicate. (C,D) Subconfluent HDFs were cultured in CM^{HDF} and either untreated or pretreated for 24 hours with 0.5 μM Na_2SeO_3 (C) or ebselen (D) before addition of 10 ng/ml rTGFβ1. TGFβ1 and selenite or ebselen were present for an additional 48 hours. The levels of cytosolic glutathione peroxidase (GPx) (C) and of αSMA (D) were detected by western blot. α-tubulin was used as loading control. Quantitative data were standardized to α-tubulin and densitometric values represent fold increase over control, which was set at 1.0. Data are representative of two independent experiments.

monolayer cultures were either preincubated for 48 hours with 0.6 nM SeP containing FCS-free DMEM before treatment with the growth factor for an additional 48 hours, or incubated with SeP over the total time period of 96 hours. In contrast to fibroblasts not treated with SeP, the rTGFβ1/ROS-mediated increase in αSMA protein levels was lowered by 41-55%, depending on the duration of SeP treatment (Fig. 2B).

As the effect of selenium supplementation suggests an antioxidant response by selenium-containing enzymes (Meewes et al., 2001), the effect of selenite on the protein level of cytosolic glutathione peroxidase was studied. Incubation of dermal fibroblasts with selenite resulted in an up to 2.3-fold increase of cytosolic GPx protein levels compared with untreated or TGFβ1-treated fibroblasts (Fig. 2C). In addition, subconfluent fibroblasts were incubated with the cytosolic GPx inhibitor mercaptosuccinate before addition of TGFβ1. Mercaptosuccinate counteracted the selenite-mediated downregulation of TGFβ1-dependent expression of αSMA, resulting in an αSMA protein level comparable with the level of TGFβ1-treated cells (data not shown).

Taken together, these data revealed a role of cytosolic GPx in protection of ROS-triggered transdifferentiation, which was confirmed using ebselen. Treatment of the fibroblasts with ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one], a cell-permeable selenoorganic compound with glutathione peroxidase activity (Müller et al., 1984; Sies, 1993), lowered the growth-factor-mediated upregulation of αSMA by 50-60% (Fig. 2D), consistent with a role of cGPx in the protection of ROS-triggered transdifferentiation.

NAC, selenite and Trolox lower TGFβ1-initiated lipid peroxidation

To characterize the type of reactive oxygen species, subconfluent HDFs were incubated with Trolox, a cell-permeant analogue of α-tocopherol, or butylated hydroxytoluene (BHT) before treatment with TGFβ1. Trolox decreased the αSMA protein level by 44% and BHT lowered it by 55%. Trolox and BHT alone had no effect on αSMA expression compared with mock-treated controls (Fig. 3A). By contrast, hydrogen peroxide and the hydroxyl radical scavengers dimethyl sulfoxide (DMSO) and mannitol did not affect the TGFβ1-dependent increase in αSMA protein amount (data not shown).

The indirect evidence for the involvement of lipid peroxidation in transdifferentiation was confirmed by direct measurements of lipid hydroperoxides (LOOH) and conjugated dienes (Kostyuk et al., 2003). Subconfluent HDF cultures were treated with rTGFβ1 for different time periods. A significant four- to fivefold increase in LOOH was detected, which peaked at 60-120 minutes compared with untreated controls. Incubation of HDFs with a combination of iron(II) sulfate and ascorbic acid 2-phosphate (Asc2P) resulted in a significant increase in intracellular LOOH content (Fig. 3B). Furthermore, subconfluent fibroblasts were preincubated with NAC, selenite or Trolox before treatment with rTGFβ1 for 1 hour. TGFβ1 increased the amount of LOOH up to 2.4-fold compared with untreated control cells. The antioxidants almost completely inhibited the growth-factor-mediated formation of LOOH (Fig. 3C). Similar results were obtained for conjugated dienes. The increase in the level of conjugated dienes up to 80% after treatment with rTGFβ1 for 2 hours was abolished by pretreatment the cells with non-toxic concentrations of NAC, selenite, or Trolox (Fig. 3D).

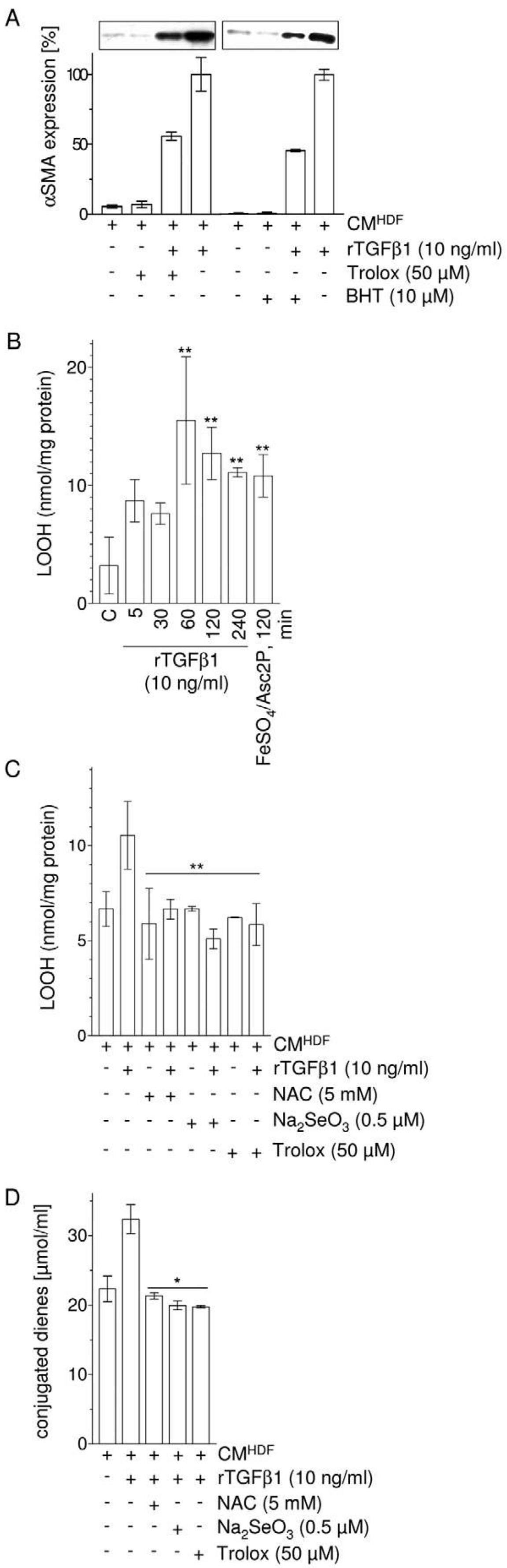


Fig. 3. Antioxidants lower TGFβ1-initiated lipid peroxidation. (A) Subconfluent HDFs were cultured in CM^{HDF} and either untreated or pretreated for 24 hours with 50 μM Trolox or 10 μM BHT before addition of 10 ng/ml rTGFβ1. TGFβ1 and the antioxidants were present for an additional 48 hours. The cell lysates were assayed by western blotting for αSMA. The experiments were performed in triplicate. (B) Time-course analysis of rTGFβ1-treated HDFs. At the indicated time points, cell lysates were prepared and the content of intracellular LOOH determined. $FeSO_4$ and Asc2P were used as a positive control. The data represent the mean ± s.e.m. of four independent experiments. ***P*<0.01 versus mock-treated controls (C) (ANOVA, Dunnett's test). (C,D) Subconfluent HDFs were incubated as described in Fig. 2A and Fig. 3A before treatment with 10 ng/ml rTGFβ1 for an additional 1 hour (see C) or 2 hours (see D) in the presence of the antioxidants. Cell lysates were prepared and subjected to LOOH measurements (see C) or detection of conjugated dienes (see D). Three independent experiments were performed. ***P*<0.01 (C) and **P*<0.05 (D) versus rTGFβ1-treated cells (ANOVA, Dunnett's test).

Involvement of PKC in TGFβ1/ROS-dependent expression of αSMA

As TGFβ1 mediates its effect on αSMA expression via ROS, potential targets of the TGFβ1-initiated signaling pathways were studied which either affect the ROS level or can be modulated by ROS. TGFβ1 activates both Smad-dependent (Heldin et al., 1997) and non-Smad downstream signaling, e.g. mitogen-activated protein kinase (MAPK) pathways (de Caestecker et al., 2000) or protein kinase C (Jinnin et al., 2005). First, the involvement of Smad2 transcription factor, a major substrate in the classical TGFβ signaling, was studied. Even though TGFβ1 initiated a time-dependent increase in Smad2 phosphorylation (Fig. 4A), a ROS-dependent phosphorylation of Smad2 was excluded by the use of antioxidants. Total Smad amounts were tested using an antibody recognizing endogenous levels of total Smad2 and Smad3 protein. The used antioxidants had no effect on the total amount of both Smad proteins (Fig. 4B).

In addition, inhibitors of signal-regulated kinase 1/2 (ERK1/2)-, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK)- and p38 MAP kinase-dependent extracellular signaling, used alone or in combination, did not affect TGFβ1-dependent expression of αSMA, indicating a MAPK-independent effect (data not shown).

Furthermore, TGFβ1 was shown to mediate biosynthesis of extracellular matrix components (Suzuki et al., 1995) as well as activation of kinases and transcription factors (Lim et al., 2005) through the involvement of protein kinase C (PKC). To study a possible involvement of PKC in TGFβ1- and ROS-triggered αSMA expression, PKC inhibitors were used. Ro 32-0432 is a selective cell-permeable PKC inhibitor, highly selective for the Ca^{2+}-dependent PKC isoforms (e.g. PKCα and PKCβ) over the Ca^{2+}-independent PKC isoforms (e.g. PKCδ and PKCε) (Haddad et al., 2005). Ro 31-8220 is a specific inhibitor which blocks all PKC isoforms, including PKCγ (Jimenez-Sainz et al., 2003). A 5.2±0.6-fold increase in αSMA protein level was measured 24 hours after rTGFβ1 treatment compared with mock-treated controls. The PKC inhibitor Ro 31-8220 completely suppressed the growth factor-mediated upregulation of αSMA, whereas the inhibitor Ro 32-0432 lowered αSMA expression by about

50% (Fig. 5A), which indicates the involvement of several PKC isoforms in TGFβ1/ROS-mediated expression of αSMA.

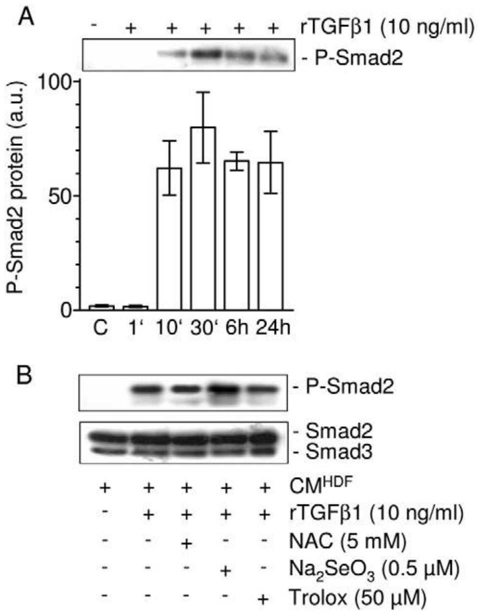
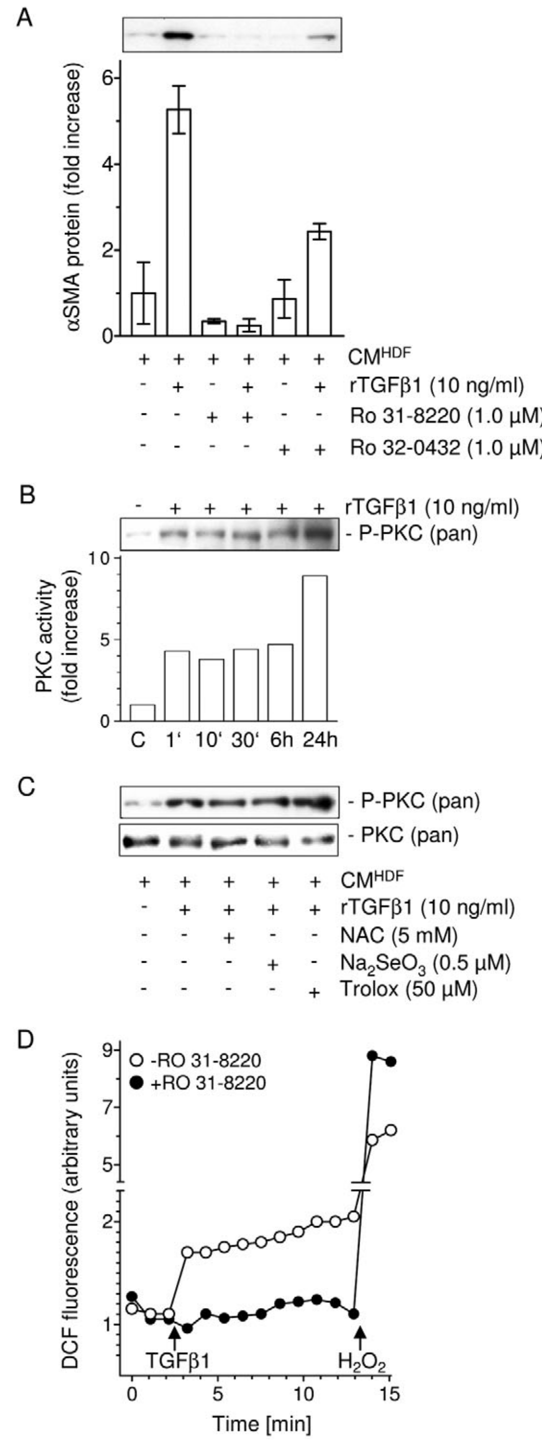


Fig. 4. ROS-mediated signaling is independent of Smad2 phosphorylation. (A) Time-course analysis of TGFβ1-initiated phosphorylation of Smad2. Subconfluent HDFs were untreated (C) or treated with 10 ng/ml rTGFβ1 CM^{HDF} for the indicated times. The cell lysates were subjected to western blot analysis for phospho-Smad2. The densitometric data (in arbitrary units, a.u.) represent means ± s.e.m. of three independent experiments. (B) Subconfluent HDFs were preincubated with the antioxidants as described (see Fig. 2A, Fig. 3A) before addition of 10 ng/ml rTGFβ1 for a further 10 minutes. The cell lysates were subjected to western blotting analysis for phospho-Smad (P-Smad2) and total Smad2/3. Total Smad2/3 was also used as a loading control. Data shown are representative of three independent experiments.

Fig. 5. Involvement of PKC in TGFβ1/ROS-dependent expression of αSMA. (A) Subconfluent fibroblasts in CM^{HDF} were preincubated with the PKC inhibitors Ro 31-8220 and Ro 32-0432 for 1 hour before treatment with 10 ng/ml rTGFβ1 in combination with each inhibitor. Expression of αSMA was detected by western blots. The densitometric analysis describes protein expression as fold increase over control, which was set at 1.0. The data represent the mean ± s.e.m. of three independent experiments. (B) Time-course analysis for activation of PKC was performed using subconfluent HDFs either untreated (C) or treated with 10 ng/ml rTGFβ1 CM^{HDF} for the indicated time periods. The cell lysates were subjected to western blot analysis for phospho-PKC. The image is representative of two independent experiments. The densitometric analysis represents fold increase over control (C) which was set at 1.0. (C) Subconfluent HDFs were preincubated with the antioxidants as described prior to addition of 10 ng/ml rTGFβ1 for a further 1 minute. Western blot analysis was performed for phospho-PKC and total PKC. Total PKC bands indicate the loading control. Data shown are representative of three independent experiments. (D) Subconfluent HDFs were preincubated with PKC inhibitor RO 31-8220 for 1 hour (closed circles) before treatment with rTGFβ1 in CM^{HDF} or 1 mM H_2O_2 for the indicated time. Increase of DCF fluorescence was followed over 15 minutes versus untreated controls (open circle). The experiments were performed in duplicate. Arrows indicate addition of rTGFβ1 or H_2O_2.

To study an interaction between ROS and PKC activation in the context of the transdifferentiation process, we performed time course analysis for phosphorylation of PKC, a marker for PKC activity (Lin et al., 2004). The phosphospecific PKC (pan) antibody detects endogenous levels of PKCα, βI, βII, γ, δ, ϵ, η, and θ isoforms when phosphorylated at a residue homologous to Thr514 of human PKCγ. A rapid and 4.3-fold increase in PKC phosphorylation was detected at 1 minute after treatment with rTGFβ1, which was maintained during the studied time, peaking at 24 hours (Fig. 5B). To check a



potential effect of antioxidants on phosphorylation of PKC, subconfluent HDFs were preincubated with NAC, selenite or Trolox before treatment with rTGFβ1 for 1 minute. Compared with untreated controls, the phospho-PKC signal was significantly increased after treatment with rTGFβ1 alone as well as in combination with NAC, selenite, or Trolox, indicating that activation of PKC is an upstream event compared with the generation of ROS. Total PKC amounts were detected by a panspecific antibody recognizing the conventional PKC isoforms α, β and γ. It became apparent that the total amount of PKC seems to be unaffected by TGFβ1 and the antioxidants (Fig. 5C).

Previous data suggest involvement of PKC in the increase in intracellular ROS levels. Time-course analysis of ROS generation after treatment of subconfluent HDFswith rTGFβ1 was performed (Fig. 5D). Incubation with the growth factor resulted in a significant increase in dichlorofluorescein (DCF) fluorescence which was maintained over the studied time range. A non-toxic concentration of 1 mM H_2O_2, used as a control, further increased the intracellular ROS level. Preincubation of HDFs with a non-toxic concentration of the most effective PKC inhibitor Ro 31-8220 (see Fig. 4A) before TGFβ1 stimulation prevented the growth-factor-initiated increase in the ROS level, indicating that generation of elevated ROS levels is downstream of activation of PKC and is affected by PKC. H_2O_2 treatment of cells, preincubated with the PKC inhibitor and rTGFβ, resulted in a significant increase in DCF fluorescence (Fig. 5D).

TGFβ1-mediated transdifferentiation in dermal and skin equivalents is inhibited by antioxidants

Three-dimensional dermal (DE) and skin equivalents (SE), which resemble the skin in vivo (Schlotmann et al., 2001), were used to exclude an artificial effect of ROS due to cells in monolayer cultures. The occurrence of myofibroblasts is characterized by their capability to contract the free-floating collagen gel/DE. A decrease in the area and diameter of the DE is inversely proportional to the increase in the number of myofibroblasts (Arora and McCulloch, 1994; Lijnen et al., 2003). Compared with the collagen lattices of untreated (Fig. 6Aa) or NAC-treated fibroblasts (c), the diameter of the lattices treated with TGFβ1 (b) was significantly lowered after 4 days of contraction, reflecting the existence of myofibroblasts. This was confirmed by an increase in αSMA expression (Fig. 6A). Preincubation of the fibroblasts located in the collagen gels

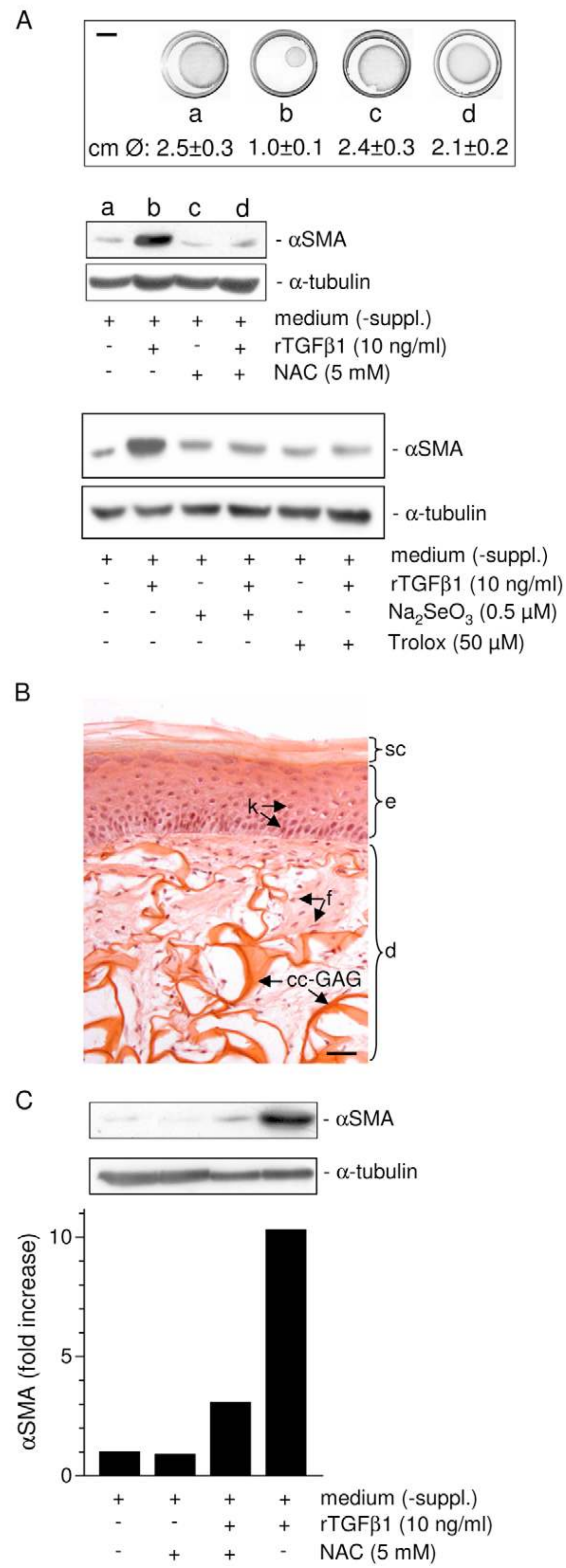


Fig. 6. Antioxidants inhibit TGFβ1-mediated transdifferentiation in dermal and skin equivalents. (A) Fibroblasts seeded for 2 days in the dermal equivalent (DE) were untreated or treated with NAC, selenite or Trolox before stimulation with rTGFβ1. After a further 48 hours, the collagen gel was dissolved with collagenase, the cells lysed and subjected to western blot analysis for αSMA. Detection of α-tubulin confirmed equal loading. The diameter (in cm) of the contracted or non-contracted collagen lattices was used as a measure of the contractile force of the cells. Three independent experiments were performed. Bar, 1 cm. (B) Histological structure of a skin equivalent (HE staining). cc-GAG, collagen-chitosan-glycosaminoglycan; d, dermis; e, epidermis; f, fibroblasts; k, normal human epidermal keratinocytes; sc, stratum corneum. Bar, 25 μm. (C) Skin equivalents were incubated for 3 days with 10 ng/ml rTGFβ1 keratinocyte-SFM medium (without supplements) or in combination with 5 mM NAC. After dispase II treatment, the dermis was homogenized and 50 μl clear lysate or sample subjected to western blot analysis. α-tubulin was used as a loading control. Quantitative data were standardized to α-tubulin and densitometric values represent fold increase over the control, which was set at 1.0. The image is representative of two independent experiments.

with NAC before TGFβ1 treatment (Fig. 6Ad) resulted in marginal contraction of the collagen lattices compared with untreated controls, which corresponds with a significantly lower expression of αSMA. These data were confirmed by preincubation of the collagen-located fibroblasts with selenite or Trolox. Again, both substances almost completely abrogated the myofibroblast-mediated contraction of the collagen lattices (data not shown), corresponding with a lowered αSMA expression compared with cells treated with TGFβ1 alone (Fig. 6A).

In addition, data were verified with the application of complete in vitro skin. Normal human skin characteristics were apparent in paraffin sections of skin equivalents stained with hematoxylin-eosin (HE) (Fig. 6B), which is in line with previously published data (Schlotmann et al., 2001). The SEs were incubated with rTGFβ1 alone or in combination with NAC for 3 days before preparation of dermal lysates for western blotting (Fig. 6C). A 10.3-fold increase in αSMA expression was detected in TGFβ1-treated SEs. NAC prevented the increase in αSMA protein amount by about 69%. NAC alone had no effect on αSMA expression.

Taken together, the data obtained with the dermis and skin equivalents agree with the data from the monolayer cell cultures, indicating that ROS are key regulators in TGFβ1-mediated fibroblast-to-myofibroblast transition in a more complex system resembling human skin.

Prevention of transdifferentiation by antioxidants inhibits the myofibroblast-mediated increase in tumor invasion in vitro

Myofibroblasts were found at the invasion front of some tumors (de Wever and Mareel, 2002), suggesting that myofibroblasts are involved in processes of tumor invasion and metastasis. Here, the hypothesis was tested that the invasive capacity of tumor cells may be modulated by antioxidant-dependent inhibition of myofibroblast formation. The formation of myofibroblasts was prevented by treatment of the subconfluent HDF cultures in $CM^{HDF,TGF}$ with NAC, selenite or Trolox. Twenty-four hours after treatment of HDFs with $CM^{HDF,TGF,antioxidant}$, the medium was replaced by serum-free DMEM for an additional 48 hours. These media ($CM^{-MF(NAC)}$, $CM^{-MF(selenite)}$, $CM^{-MF(Trolox)}$) were used for invasion assays (Fig. 7A). Compared with the medium from untreated cells (Fig. 7A CM^{HDF} and inset b), conditioned medium from myofibroblasts (Fig. 7A CM^{MF} and inset a) led to a 2.5- to 6.6-fold increase in the invasive capacity of SCL-1 tumor cells. CM^{SCL} showed the lowest chemoattractive effect on the tumor cells. $CM^{-MF(NAC)}$, $CM^{-MF(selenite)}$ and $CM^{-MF(Trolox)}$ resulted in a 84-90% lowered invasive capacity of the squamous tumor cells compared with CM^{MF}, suggesting that antioxidants play a role in prevention of myofibroblasts, and, subsequently, to lower invasion of tumor cells. Fig. 7Aa indicates collective cell motility (dotted area) which involves the movement of whole clusters of tumor cells as documented in vivo for breast, colon, and other types of carcinomas (Nabeshima et al., 1999; Sahai, 2005). Preincubation of SCL-1 cells as well as the carcinoma cell line A431 [European Collection of Cell Cultures (ECACC), Sigma] and the malignant melanoma cell line A375 (ECACC) with the antioxidants had no effect on invasive capacity compared with untreated tumor cells (data not shown).

We focused on changes in the release of cytokines and growth factors during fibroblast-to-myofibroblast transdifferentiation. Using peptide arrays for CM^{HDF} and CM^{MF}, interleukin-6 (IL-6), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) were identified to be the most prominent protein spots of CM^{MF} compared with CM^{HDF} (Fig. 7B). Compared with untreated HDFs, TGFβ1-generated myofibroblasts showed a 3.1-, 13.9- and 4.5-fold increase in VEGF, HGF and IL-6, respectively, which was almost completely abrogated by treatment of the HDFs with TGFβ1 in combination with the antioxidants NAC, selenite or Trolox (Fig. 7C). Neutralizing antibodies were used alone or in combination to modulate the chemoattractive efficacy of CM^{MF}. Excess of IL-6, HGF or VEGF neutralizing antibodies alone resulted in an slightly lowered invasive capacity of SCL-1 cells. A combination of anti-HGF and anti-IL-6 or the three neutralizing antibodies significantly downregulated the invasiveness of the tumor cells to 21% or 14% (Fig. 7D).

Discussion

Among the molecular mechanisms underlying signal transduction induced by ROS, oxidative modification of proteins and alteration in the intracellular redox state appear to be favored in the current literature. Cytokines and growth factors are known to generate ROS in non-phagocytic cells, affecting signaling components and transcription factors (Finkel, 1998; Thannickal and Fanburg, 2000). Protein modification may occur on redox-sensitive amino acids such as Cys (Herrlich and Bohmer, 2000; Jacob et al., 2003).

Lipid peroxidation and expression of αSMA

Stimulation of human lung fibroblasts with TGFβ1 results in a transient burst of ROS that regulate downstream events such as Ca^{2+} influx, MAPK activation and phosphorylation-dependent activation of activating protein-1 (AP-1) (Junn et al., 2000). Furthermore in a mouse osteoblastic cell line, TGFβ1 initiated transcription of the early growth response-1 (egr-1) gene mediated by hydrogen peroxide and redox (Ohba et al., 1994). In the present study, antioxidants downregulated TGFβ1-dependent expression of αSMA, compatible with the involvement of ROS. BHT and the water-soluble vitamin E derivative Trolox downregulated expression of αSMA, suggesting a role for lipid peroxidation. In fact, there is an increase in lipid hydroperoxides and conjugated dienes in dermal fibroblasts after treatment with TGFβ1 (Fig. 3), which is in line with earlier findings of the TGFβ1-dependent increase in lipid peroxidation induced collagen synthesis (Geesin et al., 1991). Lipid peroxidation is also involved in expression of matrix metalloproteinases (Brenneisen et al., 1998), which belong to the group of late-responsive genes, as does αSMA.

The molecular mechanism underlying lipid hydroperoxide-triggered signaling is being studied. On the one hand, peroxidative degradation of unsaturated fatty acids yields the electrophilic aldehyde 4-hydroxy-2-nonenal (4-HNE) as a major product, which can directly form stable adducts with nucleophilic amino acids such as Cys, His and Lys in receptors, downstream signaling components and transcription factors, thus affecting their activity (Petersen and Doorn, 2004). On the other hand, reactive aldehydes (e.g. 4-HNE, malondialdehyde, glyoxal) may themselves induce a specific program of gene expression, known as the cellular stress response (Uchida et al.,

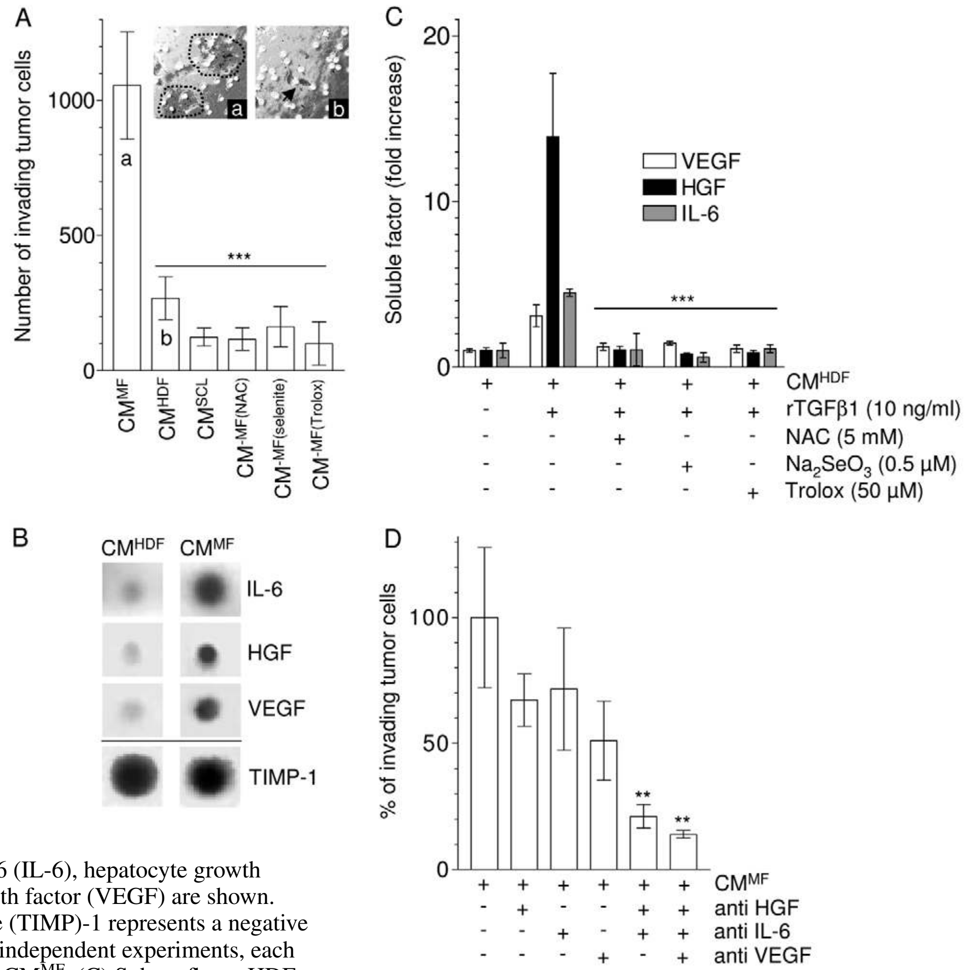


Fig. 7. Prevention of transdifferentiation lowers the invasive capacity of tumor cells. (A) Conditioned media of HDFs (CM^{HDF}), myofibroblasts (CM^{MF}), SCL-1 cells (CM^{SCL}) and HDF, which were cultured in CM^{HDF} and treated with rTGFβ1 and antioxidants ($CM^{MF(antioxidant)}$) were used for the invasion assays based on matrigel-coated transwells as described in the Materials and Methods. The total number of tumor cells migrating towards the chemoattractive media over a 72 hour time period is a measure of the invasive capacity. The data represent the mean ± s.e.m. of five independent experiments.The insets show Coomassie Blue-stained tumor cells on the lower side of the cell culture insert, migrating either as a cluster of cells (a, dotted lines) or as single cells (b, arrow head). ***P<0.001 versus CM^{MF} (ANOVA, Dunnett's test). (B) Human cytokine antibody arrays were used to compare the pattern of secreted growth factors and cytokines in the conditioned media (CM) of human dermal fibroblasts (HDF) and myofibroblasts (MF), which were collected after a 48 hour period of secretion. The most prominent signals representing interleukin-6 (IL-6), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) are shown. Tissue inhibitor of matrix metalloproteinase (TIMP)-1 represents a negative control. The image is representative of two independent experiments, each experiment is a comparison of CM^{HDF} with CM^{MF}. (C) Subconfluent HDFs were cultured in CM^{HDF} and either untreated or treated with antioxidants before addition of 10 ng/ml rTGFβ1 for an additional 24 hours. Thereafter, the medium was replaced by fresh medium (without TGFβ1 and antioxidants) and after a further 48 hours, the conditioned medium was collected and subjected to VEGF, HGF and IL-6 ELISA. ***P<0.001 versus untreated CM^{MF} (ANOVA, Dunnett's test). (D) Conditioned medium from myofibroblasts (CM^{MF}) was untreated or treated with 5 μg/ml neutralizing antibody medium, and the total number of migrating SCL-1 tumor cells is a measure of the chemoattractive capacity of antibody-treated conditioned medium compared with untreated CM^{MF}, which was set at 100%. Three independent experiments (mean ± s.e.m.) were performed. **P<0.01 versus untreated CM^{MF} (ANOVA, Dunnett's test).

1999). In that context, a nuclear localization of 4-HNE was found in cells of a macrophage line, subsequently modulating gene expression (Chiarpotto et al., 2002). A 4-HNE-dependent enhancement of apoptosis in colon cancer cells was initiated by TGFβ1 (Zanetti et al., 2003). We hypothesize that, in the model of TGFβ1/ROS-dependent transdifferentiation (Fig. 8), products of lipid peroxidation affect ROS-sensitive components of the signaling cascade, leading to αSMA expression.

PKC, ROS, and generation of myofibroblasts

Inhibitors of PKC mediated both downregulation of TGFβ1-initiated expression of αSMA and the PKC-triggered increase in intracellular ROS levels (Fig. 5), indicating that PKC is pivotal for transdifferentiation of dermal fibroblasts to myofibroblasts. In line with this observation, TGFβ1 stimulated the induction of α2(I) collagen in human dermal fibroblasts (Jinnin et al., 2005) and of α1(I) collagen in human pulmonary fibroblasts (Zhang et al., 2004) through active PKCδ. Furthermore, Gao et al. (Gao et al., 2003) showed that differentiation of rat aortic fibroblasts to myofibroblasts was inhibited by the PKC inhibitor calphostin C. Differentiation was abolished by depleting the PKCα isoform by transfection with antisense PKCα oligonucleotides. Rheumatoid synovial fibroblasts, stimulated with phorbol 12-myristate 13-acetate (PMA), produced oxyradicals through PKC-mediated activation of membrane-associated NAD(P)H oxidase (Tanabe et al., 1997). As the NAD(P)H oxidase inhibitor apocynin prevented TGFβ1-dependent αSMA expression (B.C., unpublished data), we speculate that PKC-mediated activation of NADPH oxidase results in an increase in reactive oxygen species. It was reported that PKC membrane translocation and activation mediate lipid peroxidation in cultured hepatocytes (von Ruecker et al., 1989).

Tumor invasion, soluble factors and stromal therapy

Chemo- and radiotherapeutic tumor treatment of tumor cells is accompanied by serious side effects such as rash, diarrhea, interstitial lung disease, and pneumonitis (Thomas et al., 2000;

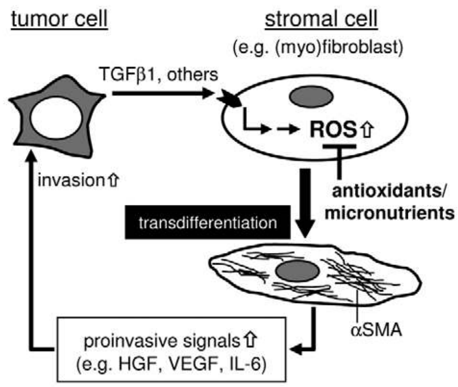


Fig. 8. Novel concept of chemoprevention of tumor progression. Tumor cells release growth factors and cytokines, e.g. TGFβ1, which initiate ROS-dependent changes of gene expression in stromal cells through receptors, resulting in transdifferentiation and release of proinvasive signals. Proinvasive signals stimulate the invasive capacity of cancer cells. These signals are lowered or prevented by treatment of stromal cells with antioxidants and/or micronutrients, finally preventing the generation of tumor-educated myofibroblasts and leading to attenuation of tumor invasion.

Birnbaum and Ready, 2005). A further problem in the successful treatment of tumor invasion is the heterogeneity of the cell population within a tumor tissue and their diverse susceptibility for therapeutic agents, overall depending on the genomic instability of the cancer cells (Tozeren et al., 2005).

As stromal cells reveal genomic stability within the tumor-host microenvironment, stromal therapy could emerge as a new strategy to combat invasion and metastatic spread. But how do we handle stromal therapy? In the present study, increased levels of reactive oxygen species account for the appearance of myofibroblasts, whereas antioxidants prevented the expression of αSMA. The fibroblastic cell type was maintained and, subsequently, the invasive capacity of tumor cells was attenuated. As a consequence of transdifferentiation, we identified significantly increased amounts of HGF, IL-6 and VEGF in tumor invasion-stimulating supernatants of myofibroblasts (Fig. 7).

HGF is a multifunctional factor acting as mitogen, motogen and morphogen for many cell types (Nakamura et al., 1989), whereas the pleiotropic cytokine IL-6 is involved in physiological and pathophysiological processes such as tumor progression (Lazar-Molnar et al., 2000). VEGF, a major mediator of angiogenesis, is associated with tumor invasion and is expressed by keratinocytes and dermal fibroblasts (Sauter et al., 1999; Detmar et al., 2000).

TGFβ1 was shown earlier to be a potent inducer of IL-6 mRNA and protein in primary human lung fibroblasts (Eickelberg et al., 1999) and of VEGF in human dermal fibroblasts (Trompezinski et al., 2000). Furthermore, tumor-derived TGFβ1 induced a myofibroblast-like phenotype of primary oral fibroblasts, which, in turn, secreted significantly higher levels of HGF (Lewis et al., 2004). HGF produced by myofibroblasts provided proinvasive signals to human colon cancer cells through cMet and/or Rac-dependent signaling (de Wever et al., 2004). In addition, both IL-6 (Arihiro et al., 2000) and VEGF (Mayr-Wohlfart et al., 2002) were shown to facilitate chemotactic motility of breast carcinoma cell lines and primary human osteoblasts, respectively. Furthermore, significantly higher circulating serum levels of VEGF, HGF and IL-6 were measured in patients with multiple myeloma compared with healthy subjects. HGF, VEGF and IL-6 appear to play pivotal roles in tumor development, even though, as shown in our study, the synergistic effect on tumor invasion is rather more dramatic than the effect of a single application.

Taken together, TGFβ1 increased the ROS level in stromal fibroblasts, which initiated the mesenchymal-mesenchymal transition and concomitant changes of gene expression, ultimately resulting in proinvasive signals for migrating tumor cells (Fig. 8). TGFβ1 therefore is considered to play a major role in the generation of 'tumor-educated' fibroblasts, namely myofibroblasts. The approach to prevent formation of myofibroblasts by the use of antioxidants and micronutrients may therefore contribute to anti-invasive and antimetastatic strategies.

Materials and Methods

Materials

Cell culture media [Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, keratinocyte-SFM medium plus supplements], and *Clostridium histolyticum* collagenase (235 U/mg) were purchased from Invitrogen (Karlsruhe, Germany) and the defined fetal calf serum (FCS gold) was from PAA Laboratories (Linz, Austria). All chemicals including protease as well as phosphatase inhibitor cocktail 1 and 2 were obtained from Sigma (Taufkirchen, Germany) unless otherwise stated. The Vivaspin 15R concentrator columns were delivered by Vivascience (Hannover, Germany). The protein assay kit (Bio-Rad DC, detergent compatible) was from Bio-Rad Laboratories (München, Germany). The lipid hydroperoxidation kit was from Cayman Chemical (Grünberg, Germany). The RayBio® Human Cytokine Antibody Array V kit as well as the VEGF and IL-6 ELISA kits were purchased from Hölzel Diagnostika (Cologne, Germany). N-acetyl-L-cysteine (NAC), sodium selenite and the selective protein kinase C (PKC) inhibitors Ro-32-0432 and Ro 31-8220 were from Merck Biosciences (Bad Soden, Germany). Matrigel and polycarbonate cell culture inserts (6.5 mm diameter, 8 μm pore size) were used from BD Biosciences (Heidelberg, Germany). The enhanced chemiluminescence system (SuperSignal West Femto Maximum Sensitivity Substrate) was supplied by Pierce (Bonn, Germany). The dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was supplied by MoBiTec (Göttingen, Germany), the enzyme dispase II and type I collagen (rat tail tendon) was from Roche Diagnostics (Penzberg, Germany). Monoclonal mouse antibody raised against human αSMA and α-tubulin were supplied by Sigma. Polyclonal rabbit anti human phospho-PKC (pan, Thr514) and phospho-Smad2 (Ser465/467) antibodies as well as anti-human Smad2 antibody were from New England Biolabs (Frankfurt a.M., Germany), while rabbit anti-human PKC (pan, α, β, γ) antibody was from Biomol (Hamburg, Germany) and rabbit anti-human glutathione peroxidase antibody from LabFrontier (Seoul, South Korea). The following secondary antibodies were used: polyclonal horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (DAKO, Glostrup, Denmark), Alexa Fluor 488-coupled goat anti-mouse IgG antibody (H+L) (MoBiTec) and goat anti-rabbit IgG antibody (Sigma). Recombinant human TGFβ1 (rTGFβ1) and the HGF ELISA kit as well as polyclonal goat anti-human HGF, VEGF and IL-6 neutralizing antibodies were from R&D Systems (Wiesbaden, Germany).

Cell culture

Human dermal fibroblasts (HDF) were established by outgrowth from foreskin biopsies of healthy human donors with an age of 3-6 years. Cells were used in passages 2-11, corresponding to cumulative population doubling levels of 3-23 (Bayreuther et al., 1992). Dermal fibroblasts and the SCC line SCL-1, originally derived from the face of a 74-year-old woman (Boukamp et al., 1982) (generously provided by Prof. Dr Norbert Fusenig, DKFZ Heidelberg, Germany), were cultured as described (Stuhlmann et al., 2003). For co-cultures of HDF and SCL-1 cells, the cells were grown on 3.5-cm tissue culture dishes at a ratio (HDF:SCL-1) of 1:1. Myofibroblasts (MF) were prepared by treatment of HDFs with different concentrations of recombinant TGFβ1 (1-10 ng/ml) for 24-48 hours in HDF conditioned medium (CM).

The human hepatocellular carcinoma cell line HepG2 (Hill et al., 1996) was grown in RPMI-1640 medium containing 10% fetal calf serum (FCS) and the supplements in 175 cm^2 tissue culture flasks. At 90% confluency, the cells were cultivated in serum-free RPMI-1640 supplemented with 100 nM sodium selenite (Na_2SeO_3) for an additional 2 days. The HepG2 supernatant containing the secreted selenoprotein P was collected, the cell debris was removed by centrifugation, and the supernatant was concentrated (40×) by ultrafiltration using Vivaspin 15R concentrator with a 30 kDa cut-off hydrosart membrane.

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Preparation of dermal and skin equivalents

Three-dimensional collagen lattices were prepared as described (Mauch et al., 1988) with minor modifications. Briefly, type I collagen from rat tail tendon was redissolved at 3.2 mg/ml in sterile 0.2% acetic acid. Human dermal fibroblasts were seeded at 1.25×10^5 cells/ml into a NaOH-neutralized solution containing 0.8 mg collagen/ml 1× DMEM with 5% FCS and grown for 24 hours at 37°C in 3.5-cm-diameter uncoated bacterial dishes. Cells in that mechanically relaxed lattices were allowed to contract the gel matrix. The medium was replaced by serum-free medium or serum-free medium containing non-toxic concentrations of NAC, Trolox or selenite, and the collagen lattices incubated for a further 24 hours before addition of recombinant TGFβ1. After 48 hours, each collagen lattice was photographed and the diameter (in cm) used as a measure of the contractile force of the (myo)fibroblasts. Thereafter, the collagen lattice was washed in phosphate-buffered saline and digested with 3 mg/ml *Clostridium histolyticum* collagenase PBS for 30-45 minutes at 37°C. After centrifugation, the cells were lysed and subjected to western blot analysis.

The skin equivalents were prepared as previously described (Damour et al., 1994; Schlotmann et al., 2001). Briefly, a suspension of 2×10^5 dermal fibroblasts/cm^2 was added in each well of a 24-well plate on top of a collagen-chitosan-glycosaminoglycan (cc-GAG) biopolymer and the dermal equivalent (DE) was cultured for 14 days in DMEM plus 10% FCS containing 50 μg/ml ascorbic acid under submerged conditions in a humidified atmosphere. The medium was changed every 2 days. Normal human epidermal keratinocytes, seeded at a density of 2×10^5 cells/cm^2 on a 14-day-old DE, were cultured in keratinocyte SFM medium with supplements (human epidermal growth factor, bovine pituitary extract) and 50 μg/ml ascorbic acid for an additional 7 days. Thereafter, the skin equivalent (SE) was raised at the air-liquid interface for further 14 days in keratinocyte SFM medium/supplements and ascorbic acid to allow keratinocytes to stratify and differentiate until horny layers formed, while the medium was changed every 2 days. SE was fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 6 μm thickness were stained using hematoxylin-eosin (HE).

Skin equivalents were incubated for 3 days with recombinant TGFβ1 or in combination with 5 mM NAC. To separate reconstructed dermis from epidermis for preparation of lysates, SEs were incubated for 1 hour in prewarmed dispase II solution (2.4 U/ml) at 37°C. The (dermis) samples were incubated for 30 minutes at 4°C in 1% SDS in Tris/EDTA (250 μl/sample) supplemented with 1:1000 diluted protease and phosphatase inhibitors. Thereafter, the samples were homogenized with a Dounce homogenizer (Braun, Melsungen, Germany) followed by sonication for 10 seconds. The samples were centrifuged at 4°C and supernatants were subjected to western blot analysis.

Preparation of conditioned media

Conditioned medium was obtained from SCL-1 cells (CM^{SCL}), human dermal fibroblasts (CM^{HDF}) and myofibroblasts (CM^{MF}). For this, seeded 1×10^6 SCL-1 cells were grown to subconfluence (~70% confluence) and 1.5×10^6 HDF cells to confluence in 175-cm^2 culture flasks to get identical cell numbers. The serum-containing medium was removed, and after washing in phosphate-buffered saline (PBS) the cells were incubated for further 48 hours in 15 ml serum-free DMEM before collection of the control conditioned medium. CM for invasion assay was obtained from myofibroblasts. To transdifferentiate fibroblasts to myofibroblasts 3×10^4 HDFs were grown to subconfluence (~80% confluence) on 3.5-cm diameter tissue culture dishes. After removal of serum-containing medium, subconfluent HDFs were cultured in CM^{HDF} and either untreated or pretreated for 4 hours with 5.0 mM NAC, for 24 hours with 0.5 μM Na_2SeO_3 or for 24 hours with 50 μM Trolox before addition of 10 ng rTGFβ1/ml. TGFβ1 alone (CM^{HDF,TGF}) or in combination with the antioxidants (CM^{HDF,TGF,antioxidants}) was present for additional 24 or 48 hours. CM^{HDF,TGF} or CM^{HDF,TGF,antioxidants} were replaced by 1 ml serum-free medium and the cells incubated for additional 48 hours. Thereafter the CM of myofibroblasts (CM^{MF}) and non-transdifferentiated fibroblasts (CM$^{-MF(antioxidant)}$) were collected and clarified by centrifugation at 1250 ***g***. Conditioned media were used fresh or stored at –20°C for at the most 2 weeks before use.

Fluorimetric Se determination

The selenium content of the concentrated HepG2 supernatants was determined by a fluorimetric assay as described (Schomburg et al., 2003), using 150 μl of supernatant. The selenium concentration was calculated according to a standard curve of serial dilutions of Na_2SeO_3 in water (1 nM to 10 μM). The selenoprotein P concentration was calculated from the selenium content of the sample, assuming that the selenoprotein P molecule contains an average of 10 Se per molecule as selenocysteine.

Measurement of intracellular ROS

The intracellular ROS level was measured as described (Stuhlmann et al., 2004) with minor modifications. Briefly, subconfluent fibroblast monolayer cultures were loaded with 20 μM H_2DCF-DA in PSG buffer (100 mM KH_2PO4, 10 mM NaCl and 5 mM glucose, pH 7.4) for 15 minutes in the dark. After washing three times with PSG buffer, the loaded cells were treated with 10 ng/ml rTGFβ1 PSG. To show a PKC-mediated increase in intracellular ROS level, subconfluent fibroblasts were incubated with 1.0 μM PKC inhibitor Ro 31-8220 for 1 hour before treatment with the growth factor. ROS generation was detected as a result of the oxidation of H_2DCF and the fluorescence (excitation 488 nm; emission 515-540 nm), given in arbitrary units, was followed with a Zeiss Axiovert fluorescent microscope with a charge-coupled device (CCD) camera (ORCA II, Hamamatsu, Herrsching, Germany) for 15 minutes.

Lipid peroxidation assay

Quantitative determination of lipid hydroperoxides in the rTGFβ1-treated HDF cells was performed according to the manufacturer's instructions with minor modifications. Briefly, subconfluent HDFs in CM^{HDF}/5 μM EDTA were treated for 5, 30, 60, 120 and 240 minutes with 10 ng rTGFβ1/ml, washed with PBS/5 μM EDTA, lysed with 500 μl H_2O containing 10 μM butylated hydroxytoluene (BHT), and subjected to LOOH measurements. As a positive control, HDFs were treated with a combination of 1.0 mM Fe(II)sulfate/250 μM ascorbic acid 2-phosphate (Asc2P) for 120 minutes. In addition, the cells were preincubated with CM^{HDF} alone or in combination with antioxidants for 24 hours before treatment with rTGFβ1 for the indicated time. The absorbance was measured at 500 nm. The amount of lipid hydroperoxides was calculated from a standard curve ranging from 0-5 nM. Extracted conjugated dienes of the chloroform layer were also measured at 234 nm. The amount of conjugated dienes was determined by the extinction coefficient (ϵ_{234}=29,500 M^{-1} cm^{-1}).

Immunocytochemistry

Immunostaining was performed as described elsewhere (Stuhlmann et al., 2003). Briefly, subconfluent co-cultures and HDF monolayer cultures were grown in DMEM plus 10% FCS on coverslips in 3.5-cm diameter tissue culture dishes before use. Cells were incubated with monoclonal αSMA antibody diluted 1:1000 in 1% (v/v) NGS/PBS overnight at 4°C. After washing, the cells were incubated with an Alexa Fluor 488-coupled goat anti-mouse IgG (diluted 1:1000 in PBS) for 1 hour at room temperature. DAPI staining was performed as described (Stuhlmann et al., 2003). Images were taken with a Zeiss Axiovert fluorescence microscope with a CCD camera (ORCA II, Hamamatsu, Herrsching, Germany). The percentage of αSMA-positive cells was determined by counting 25 fields per dish and calculated as the number of αSMA-positive cells per total cells/field.

SDS-PAGE and western blotting

SDS-PAGE was performed according to the standard protocols published elsewhere (Laemmli, 1970) with minor modifications. Briefly, cells were lysed after incubation with rTGFβ1 in 2× SDS-PAGE buffer (125 mM Tris-HCl, 4% w/v glycerol, 100 mM dithiothreitol, pH 6.8). After sonication, the protein concentration was determined by using a modified Lowry method (Bio-Rad DC). Thereafter, Bromophenol Blue was added (0.1% final concentration), and after heating, the samples (5 μg total protein/lane) were applied to 10% (w/v) SDS-polyacrylamide gels. After electroblotting onto nitrocellulose membrane, immunodetection was carried out using either a 1:1000 dilution of primary antibodies (mouse monoclonal anti α-SMA and α-tubulin, rabbit polyclonal anti phospho-Smad2, total Smad2/3, rabbit polyclonal anti GPx, and anti phospho-PKC) or a 1:500 dilution for anti PKC and a 1:20,000 dilution of anti mouse or 1:2000 dilution of anti rabbit secondary antibody conjugated to HRP. Antigen-antibody complexes were visualized by an enhanced chemiluminescence system on BioMax Light Film (Kodak, Rochester, USA). Equal loading was checked by Coomassie Blue staining. Molecular sizes of the bands were calculated by comparison with a prestained protein marker. For quantification of the bands, the developed films were scanned by an image analysis system and analysed with the AIDA image software.

Human cytokine antibody array

A human protein cytokine array was performed according to the manufacturer's instructions. Briefly, the membranes with the spotted cytokine antibodies were blocked with a blocking buffer, and thereafter incubated with 1 ml CM^{HDF} and CM^{MF}, respectively, at room temperature for 2 hours. After washing, the membranes were treated with 1 ml of a cocktail of primary biotin-conjugated antibodies for additional 2 hours at room temperature. Thereafter, the membranes were incubated with 2 ml horseradish peroxidase-conjugated streptavidin/membrane at room temperature for 2 hours. The membranes were developed by enhanced chemiluminescence system on BioMax Light Film.

Enzyme-linked immunoassay (ELISA)

ELISAs for VEGF, HGF and IL-6 were performed according to the manufacturer's protocols. Briefly, serial dilutions of standards and samples were incubated at room temperature on 96-well immunoplates, precoated with capture antibody. After this and each subsequent step, plates were washed four times with 0.005% Tween 20 in PBS. Subsequently, plates were incubated at room temperature with a biotinylated anti-growth-factor antibody, followed by incubation with horseradish peroxidase-conjugated streptavidin. Finally, 3,3′,5,5′tetramethylbenzidine (TMB) peroxidase substrate solution was added to each well. The reaction was stopped by addition of sulphuric acid. Optical densities were read at 450 nm using a microtiter plate reader. Concentration of different cytokines to be tested in the samples were determined against standard curves using GraphPad software (San Diego, CA).

Invasion assay

Polycarbonate cell culture inserts (transwells) were overlaid with 125 μg growth factor reduced Matrigel/insert and were placed in a 24-well plate. SCL-1 tumor cells (5×10^4 cells/insert) were seeded on top of the matrigel in serum-free DMEM. CM^{MF}, CM^{HDF} and CM of antioxidant and rTGFβ1-treated HDF cells ($CM^{MF(antioxidant)}$, see above) were used as chemoattractant in the lower chamber. Furthermore, HGF-, IL-6- and VEGF-depleted CM^{MF} was used. After 72 hours at 37°C, the cells were rubbed off the upper side of the filter using cotton swabs, and the SCL-1 cells, which invaded to the lower side of the insert, were stained with Coomassie Blue solution (0.05% Coomassie Blue, 20% MeOH, 7.5% acetic acid). The number of invaded cells was estimated by counting 25 random microscopic fields/insert.

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